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DEVELOPMENT OF A THERAPEUTIC (NEUTROPHIL-BLOCKING AGENT TO AMELIORATE THE PATHOLOGY OF BACTERIAL INFLAMMATION IN CATTLE

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Development of a therapeutic (neutrophil-blocking) agent to ameliorate the pathology of bacterial inflammation in vivo (ADF Project 20010326 - PI, John R. Gordon)

(A) ABSTRACT/SUMMARY

The pathology of shipping fever bronchopneumonia associated with *Pasteurella hemolytica* infections is largely mediated by the host's overly exuberant neutrophil response to the bacterial infection. We have shown previously that secretion of the cytokine interleukin-8 (IL-8) is the host's trigger for this neutrophil response, and that by experimentally neutralizing IL-8 we can block a substantial part of *in vitro* neutrophil responses to pathogenic mediators produced within the lungs during this disease. Under ADF grant 9800236 we developed and tested a recombinant protein (bovine IL-$8_{(3-73)}$K11R/G31P [G31P]) that, *in vitro*, can block all of the ELR-CXC chemokines expressed *in vivo* during intense inflammatory responses. G31P also blocks the responses of human neutrophils to the range of ELR-CXC chemokines. Furthermore, we demonstrated that it blocks pyrexia and hemorrhagic consolidation following the induction of experimental airway endotoxemia, as well as dramatically reducing airway neutrophilic inflammation, and also that it blocks intradermal inflammatory responses to endotoxin challenge for 2-3 days following one G31P treatment. These results amply verify our hypothesis that G31P is also efficacious *in vivo*. This reagent is presently under negotiation for commercial licensing (University of Saskatchewan Technologies Inc, and Lombard Western Life Sciences).

(B) EXECUTIVE SUMMARY

Neutrophils and their mediators are critical to the elimination of bacterial infections. While the homeostatic processes that reduce host exposure to neutrophil mediators detrimental to the *host's* systems have been carefully groomed, aberrant neutrophil responses are directly relevant to, and augment greatly, the pathology of many diseases; in cattle with shipping fever pneumonia, for example, they in fact cause most of the tissue injury[1].

There are multiple molecules that stimulate neutrophil responses, including the ELR-CXC chemokines (e.g., ligands of the CXCR1 and CXCR2). The relative importance of each of these to inflammation pathology has never been systematically assessed, although in any one setting one or more has been implicated. We now have substantial evidence that the ELR-CXC chemokines occupy a keystone position in this cascade.

We previously generated an all-inclusive ELR-CXC chemokine antagonist using the CXCL8/IL-8 gene as a platform into which we introduced amino acid substitutions predicted by rational design to impact receptor affinity and signaling. We first engineered high affinity into CXCL8/IL-8, documenting CXCL$8_{(3-73)}$K11R (one of many generated) as the optimized agonist[2], then removed this molecules' receptor signaling properties without sacrificing its high receptor affinity. From two families of antagonists, we identified CXCL$8_{(3-73)}$K11R/G31P (G31P) as the optimal antagonist[3]. G31P blocks CXCL8/IL-8-dependent neutrophil chemotaxis, degranulation, and O_2^- release. It blocks ligand binding to both the CXCR1 and CXCR2, so that it is effective against CXCL1, CXCL5, and CXCL8 [3]; when titrated against its

parent chemokine, 0.5 nM G31P 50% blocks neutrophil responses to 129 nM CXCL8/IL-8 [4]. At ≤10 ng/ml, it also (75-93%) blocks responses to the neutrophil chemoattractants present in wash fluids from the lungs of cattle that have died of pneumonic pasteurellosis, as well as those from cows with endotoxin-induced mastitis; anti-IL-8 antibodies block ≈35-65% of these activities[4-6].

In the research arising from funds provided under this grant, we critically assessed the ability of G31P to ameliorate endotoxin-induced pathology *in vivo* using an intradermal endotoxin-challenge model and an airway endotoxemia model. In both systems we found that G31P had remarkable effects in terms of reducing neutrophilic inflammatory responses, but also in reducing more tangential sequelae of these responses (fever, airway hemorrhagic consolidation).

(C) TECHNICAL REPORT

BACKGROUND AND OBJECTIVES

We have shown that IL-8 is highly expressed in the lungs of ruminants (cattle or lambs) with pneumonic pasteurellosis and that antibodies to IL-8 can neutralize up to ≈65% of the neutrophil chemotactic activity of lung wash fluids harvested from these animals[4-6]. Based on this close association between this IL-8 expression and neutrophil chemoattraction and the impact of neutrophils on the pathology of this disease[1], we attempted to rescue lethally-challenged pneumonic pasteurellosis lambs by treating them with up to 80 mg of neutralizing anti-IL-8 antibodies[6] (ADF grant #96000087). We were unsuccessful in this attempt for three reasons; first, the massive challenges induced the expression of extraordinary levels of IL-8 within the lungs of these animals; second, other neutrophil chemoattractants (i.e., ENA78, GRO) are also expressed in the lungs of these animals[7]; and third, the cost of the of the antibodies was prohibitively expensive, so that we could not treat the lambs with levels of antibody which would have ensured complete IL-8 neutralization.

Under ADF grant 9800236 we designed and developed a recombinant protein (G31P) that, *in vitro*, can block all of the ELR-CXC chemokines expressed *in vivo* during intense inflammatory responses[3]. Thus, this project had one primary objective – to confirm that G31P could be efficacious against bacterial endotoxin-driven neutrophilic inflammation *in vivo*. To accomplish this, we employed two endotoxin challenge models, one intradermal and the other airway endotoxemia.

PROGRESS WITHIN THE PROJECT

METHODS

Reagents & supplies. All reagents were purchased commercially or generated in our laboratory (e.g., G31P). Animals were purchased locally (e.g., Saskatoon Auction mart)

Generation of IL-8(3-73)K11R analogues. We have reported in detail the methods for the generation and purification of G31P[3]. All G31P preparations were characterized by PAGE and

Western blotting with goat anti-bovine IL-8 antibody; each purified analogue had a molecular mass of ≈8 kD and was specifically recognized by the anti-IL-8 antibody in the Western blotting. The relative purity of each preparation was estimated by densitometric analysis of its' PAGE gel at ≥96%.

G31P blockade of endotoxin-induced neutrophilic inflammation *in vivo*.

Intradermal endotoxin challenge model. Animals were given a series of 15 h skin tests for reactivity to bacterial endotoxin challenge[4]. At day –1 each was injected intradermally with 1 μg endotoxin and 15 h later a 6 mm punch biopsy was taken of the injection site. At time 0, each animal was administered G31P (75 μg/kg) either s.c., i.v., or i.m., then immediately thereafter, and again at 33h and 58 h post-treatment, they were challenged i.d. with endotoxin.

Airway endotoxemia model. In an initial experiment, animals were challenged via the airway with 0, 5, 25, or 100 μg/kg of endotoxin. At the time of challenge, and at various times thereafter the rectal temperatures of the animals were determined using a digital thermometer. In addition, at the time of challenge the animals were injected subcutaneously (s.c.) with 0-250 μg/kg of G31P or with an equivalent volume of saline, and at 15 h they were euthanized. Peripheral blood was obtained by venipuncture to assess residual circulating neutrophil responsiveness to ELR-CXC chemokines and bronchoalveolar lavage (BAL) samples were obtained. All samples from each animal were assayed independently and either examined immediately or stored at -80°C prior to assay. The degree of hemorrhagic consolidation was assessed by computer-aided image analysis of the respiratory tree after removal from the animals post-mortem.

Neutrophil isolation, bronchoalveolar cells and fluids. Heparin-anticoagulated plasma was obtained by venipuncture, and neutrophil-enriched cell populations obtained as the high cell density fractions from Lymphocyte Separation Medium (LSM) density gradient centrifugation[3, 4]. Blood smears and cytocentrifuge slides of airway cells obtained by BAL were stained with Wright's solution and differential counts performed on 200 cells per sample[4, 8, 9]. Neutrophils recovered from the BAL fluids were also separated on LSM gradients and assessed for their responsiveness to ELR-CXC chemokines *in vivo*.

Lung & skin histopathology

Each intradermal endotoxin challenge site was biopsied 15 h post-challenge using a 6 mm biopsy punch. Biopsies of left cranial lung lobes of each animal and skin biopsies were fixed for 3 h in acid-alcohol formaldehyde and processed to 6 mm paraffin sections. For routine histopathology, the tissue sections were stained with Giemsa solution and examined in a blinded fashion at 400x magnification[10]. The numbers of neutrophils infiltrating the dermis or in the pulmonary parenchymal tissues were enumerated as noted.

Neutrophil chemotaxis assays

Microchemotaxis assays were run in duplicate modified Boyden microchemotaxis chambers using polyvinylpyrrolidone-free 5 μm pore-size polycarbonate filters[2, 3, 8, 11]. For each sample, the numbers of cells that had migrated into the membranes over 20 min were enumerated by direct counting of at least nine 40x objective fields, and the results expressed as the mean number of cells/40x field (+/- SEM). The chemoattractants included recombinant human CXCL1/GROa, CXCL5/ENA-78, or CXCL8/IL-8, or BAL fluids from the lungs of the treated animals (diluted 1:1 – 1:40), while the antagonists comprised CXCL8-specific antibodies or G31P. In some

assays we preincubated the samples with the antibodies (5 µg/ml) for 60 min on ice[12]. For assays with G31P, the inhibitor was mixed directly with the samples immediately prior to testing.

Cytokine Bioassays

IL-1 assay. The LM-1 cell, a sub-clone of the ATCC cell line D10.G4, was provided by Dr. D. Godson (Vaccine and Infectious Diseases Organization). The details of this assay have been reported previously[13]. Briefly, IL-1-starved LM-1 cells were plated in 96-well plates at $4x10^5$ cells/ml in Click's medium supplemented with 10% fetal calf serum, L-glutamine and 1% penicillin/streptomycin/fungizone, together with varying volumes of BAL fluids (1-100 µl/well) and either control or IL-1-specific antibodies (R & D Systems). After 5 days, the cell proliferative responses were assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Insoluble MTT formazan precipitates formed within the cells were solubilized with acidified isopropanol and quantified spectrophotometrically at OD_{595}. The sensitivity of this assay is ≈10 pg/ml IL-1.

IL-6 assay. The 7TD1 cells were obtained from the ATCC. The details of this assay have similarly been published[13]. Briefly, 7TD1 cells were plated in 96-well plates at $1.25x10^4$ cells/well in RPMI-10 supplemented with 10% fetal calf serum, L-glutamine and 1% penicillin/streptomycin/fungizone, together with varying volumes of BAL fluids (1-25 µl/well) and either control or IL-6-specific antibodies (R & D Systems). The proliferative responses were assessed as above for the LM-1 cells. The sensitivity of this assay is ≈25 pg/ml IL-6.

TNF assay. The details of the L929 cell cytotoxicity assay for TNF have been reported previously[14]. Briefly, varying volumes of BAL fluids were incubated overnight with sub-confluent monolayers of L929 cells in DMEM supplemented with 2.5 µg/ml of actinomycin D (in 96-well plates) and either control or TNF-specific antibodies (R & D Systems, Inc). The cytotoxicity in each well was determined by MTT uptake, as noted above, with the activity calculated using the formula:

$$\frac{(\text{mean } OD_{595} \text{ medium control wells - } OD_{595} \text{ experimental well})}{(\text{mean } OD_{595} \text{ medium control wells} - \text{mean } OD_{595} \text{ 100\% cytotoxicity wells})} \times 100$$

Statistical analyses

Multi-group data were analyzed by ANOVA and *post-hoc* Fisher protected Least Significant Difference (PLSD) testing, while two-group comparisons were made using the students t-test (two-tailed). The results are expressed as the mean +/- SEM.

RESULTS

G31P is an effective antagonist of neutrophilic inflammation in airway endotoxemia. The animals that were given bacterial endotoxin via the airway developed pyrexia within 3 h, and this response increased to a maximum at 6-8 h, then waned moderately such that at 24 h the animals were still feverish. Treatment G31P abrogated the fever response in all animals tested (Fig 1). We also

assessed the levels of proinflammatory mediators, including the endogenous pryogen IL-1 and TNF, in the airways of these animals, and found the G31P treatments markedly reduced their expression (Fig. 2), which likely explains the reduced fever responses in these animals. Neutrophils comprised 93+/-12% of the cells recovered from the BALF of cattle with advanced pneumonic pasteurellosis and similarly high levels of neutrophils were recovered from the animals with airway endotoxemia. Airway endotoxemia also induced a circulating neutrophilia in these animals (Fig. 3). G31P treatments significantly reduced the circulating and airway neutrophilia in the pulmonary endotoxemic animals (Fig. 3). The lungs of the endotoxemic animals displayed marked local hemorrhagic consolidation, and this effect was also reduced significantly by the G31P treatments (Fig. 4). As demonstrated for animals with pneumonic pasteurellosis, when tested *in vitro*, BALF samples from the airway endotoxemia animals were strongly chemotactic for neutrophils, and treatment of these BALF samples with G31P (0-10 ng/ml) reduced the neutrophil responses by up to 98%, relative to the medium controls (data not shown). Furthermore, the circulating neutrophils of the G31P-treated animals were unresponsive to ELR-CXC chemokine signaling *in vitro* (Fig 5).

G31P blocks neutrophilic inflammation in cattle challenged with bacterial endotoxin. We gave our animals a series of 15 h skin tests for reactivity to bacterial endotoxin challenge at various times before or after treatment with G31P (Fig. 6). We found that each animal responded strongly to the endotoxin challenge administered before G31P treatment, but the responses at 24 and 48 h post-treatment were markedly (≈86% at 24 h) reduced. By 72 h post-treatment the neutrophils of the calves were beginning again to respond to the endotoxin challenges, indicating that the G31P effects were transient.

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(d) Personnel: No personnel were paid from ADF funds.

(e) Equipment: No equipment was purchased with ADF funds.

(f) Project Developed Materials: No new materials were developed under this funding

(g) Project Photos: Photographs are attached above

(h) Acknowledgement

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Fig.1. Treatment with G31P abrogates the fever response to bacterial endotoxin challenge. Each animal was given E. coli endotoxin (LPS) by airway intubation at time 0 h, then either saline or G31P (100 μg/kg) at time 0 or 4 h post-LPS challenge. The body temperature was determined at various times thereafter. The LPS-challenged animals developed mild fever responses that were evident within 3 h of challenge, and increased steadily thereafter. The G31P treatments abrogated the fever responses, whether given at the time of LPS challenge or after pyrexia had begun

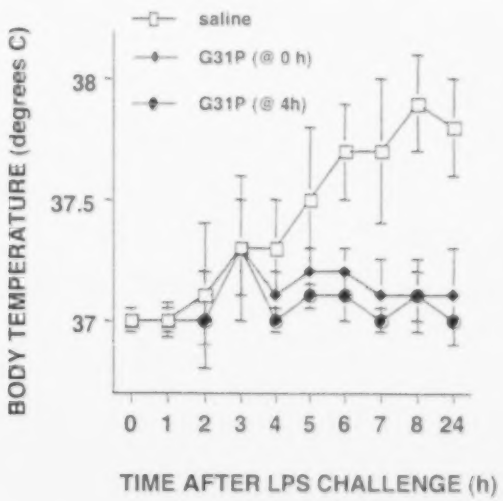


Fig.2. Treatment with G31P dramatically reduces the expression of endogenous pyrogens in the airways of animals challenged with bacterial endotoxin. Each animal was given E. coli endotoxin (LPS) as in Fig 1, and either saline or G31P (100 μg/kg) at the time of LPS challenge. The airway levels of IL-1, IL-6, and TNF were assessed by bioassay for each cytokine using bronchoalveolar lavage (BAL) fluids. The G31P treatments significantly reduced the expression of each inflammatory mediator in the endotoxemic airways.

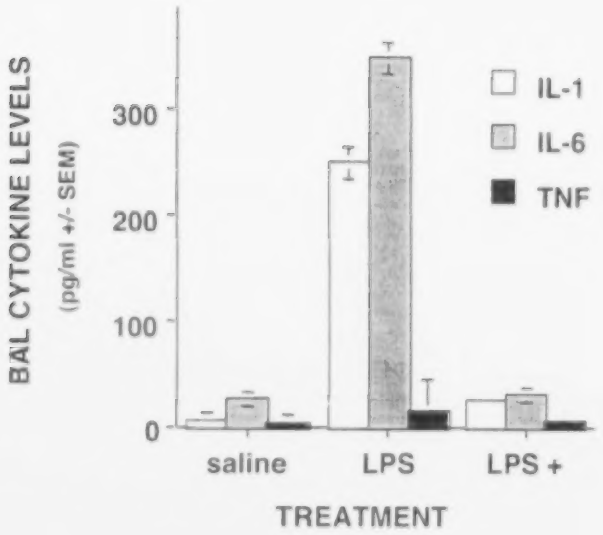


Fig.3. Treatment with G31P significantly reduces the development of circulatory and airway neutrophilia following bacterial endotoxin challenge. Each animal was given E. coli endotoxin (LPS) as in Fig 1, and either saline or G31P (100 µg/kg) immediately thereafter. The circulating neutrophil counts were determined from blood smears, while the airway neutrophilia was assessed post-mortem, from differential counts of bronchoalveolar lavage (BAL) cell cytocentrifuge preparations The LPS-challenged animals developed a mild circulating neutrophilia, as has been reported previously more multiple species, and the G31P treatments markedly ameliorated this response. Similarly, airway endotoxemia induced strong neutrophilic airway inflammation which was dramatically ameliorated by the G31P treatments

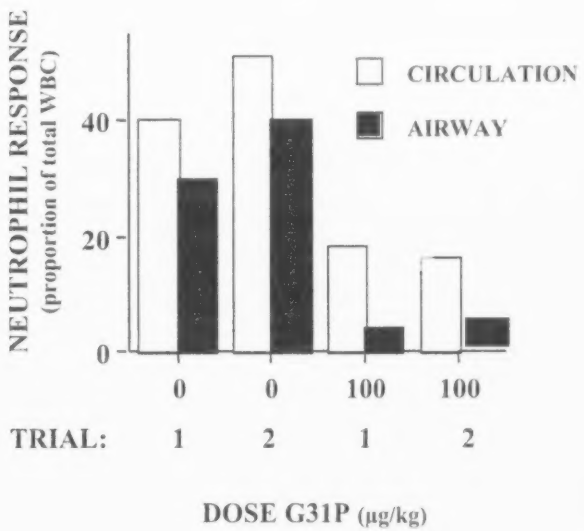
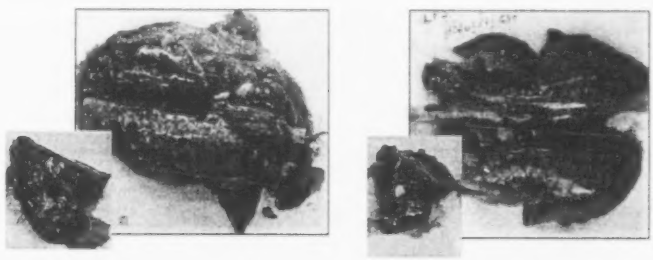


Fig.4. Treatment with G31P significantly reduces the development of pulmonary hemorrhagic responses following bacterial endotoxin challenge. Each animal was given E. coli endotoxin (LPS) as in Fig 1, and either saline or G31P (100 μg/kg) immediately thereafter. The respiratory tree was removed from each animal at post-mortem and photographed (A), then subjected to image analysis to assess the proportion of the lung surface area displaying signs of hemorrhagic pathology (B). The LPS-challenged animals developed substantial pulmonary hemorrhages, and the G31P treatments markedly ameliorated this response.

A



B

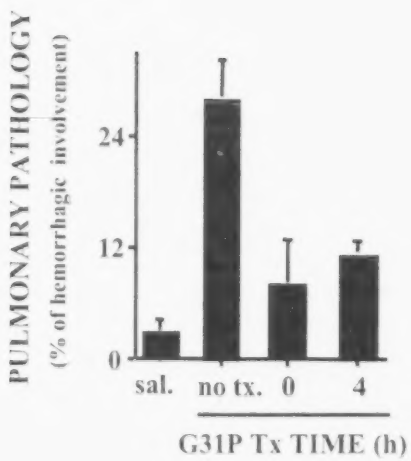


Fig. 5. The circulating neutrophils of G31P-treated animals are hyporesponsive to ELR-CXC chemokine signaling *in vitro*. Circulating neutrophils purified from the G31P-treated animals were tested for their *in vitro* responsiveness to CXCL8/IL-8 or CXCL5/ENA-78 (0-100 ng/ml) using microchemotaxis assays. The cells were entirely resistant to signaling via either the CXCR1 (CXCL8) or the CXCR2 (CXCL5)

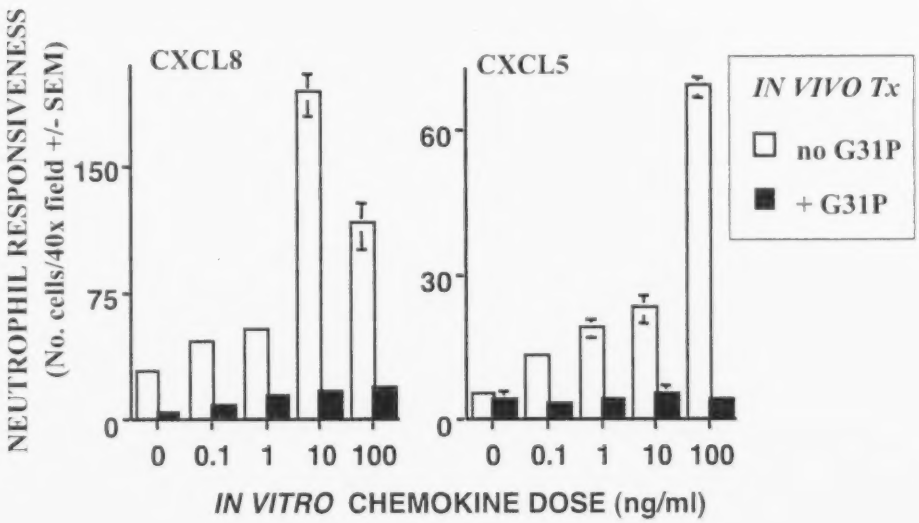


Fig.6. Treatment with G31P significantly reduces the development of dermal neutrophilic inflammation following bacterial endotoxin challenge. Each animal was injected intradermally with *E. coli* endotoxin (2.5 μg/site; LPS) at time -15h, then biopsies were taken at time 0 h and the neutrophilic inflammation was assessed from paraffin sections. At 0 h, the animals were treated subcutaneously with G31P (100 μg/kg) and at the same time challenged with LPS. The LPS challenges were repeated at 33 and 59 h. For each of challenge site, 15 h reaction biopsies were obtained. LPS-challenge induced strong neutrophilic inflammatory responses (0 h), and these were markedly, but transiently ameliorated by the G31P treatments.